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Extreme Genetic Diversity among Pirital Virus (*Arenaviridae*) Isolates from Western Venezuela

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Pirital-like virus isolates from rodents collected in a variety of habitats within a six-state area of central Venezuela were analyzed genetically by amplifying a portion of the nucleocapsid protein gene using RT-PCR. Comparisons of the sequences from 30 selected Pirital-like virus isolates demonstrated up to 26% divergence in nucleotide sequences and up to 16% divergence in deduced amino acid sequences. Within the Pirital monophyletic group, 14 distinct lineages or genotypes, differing by at least 6% in nucleotide sequences, were identified. Although sample sizes were small for some lineages, many of the different genotypes were sampled in only one region or locality, suggesting allopatric divergence. Complement fixation tests with representatives of the most divergent Pirital virus lineages failed to delineate multiple species or subtypes within the Pirital clade. These results indicate that the previously proposed 12% nucleocapsid protein amino acid sequence divergence cutoff value for delineating arenavirus species is not appropriate for the entire family. When individual clones were examined from PCR amplicons, a mean of 0.17% sequence diversity vs the consensus sequences was detected, suggesting diverse quasispecies populations within infected rodent hosts. Possible explanations for the extreme genetic diversity within and among Pirital virus populations in infected rodents are discussed.

INTRODUCTION

Viruses of the genus *Arenavirus,* family Arenaviridae, are enveloped viruses with a genome consisting of two single-stranded RNA segments, designated small (S) and large (L). Each segment contains two nonoverlapping genes arranged in an ambisense orientation (Bowen *et al.,* 1997; Southern, 1996). Based on their antigenic and phylogenetic relationships, the arenaviruses are divided into two groups or complexes: the lymphocytic choriomeningitis–Lassa complex, which includes the Old World arenaviruses; and the Tacaribe complex, which includes all of the known New World arenaviruses (Bowen *et al.,* 1997). Most of the arenaviruses have a rather restricted geographical distribution, and in some cases, they are sympatric (Table 1) (Tesh *et al.,* 1999). In general, a single vertebrate species, usually a rodent, serves as the natural reservoir host for each virus (Bowen *et al.,* 1997; Tesh *et al.,* 1999).

Genetic studies with five different arenaviruses, Lassa (Bowen *et al.,* 2000), Junin (Garcia *et al.,* 2000), Guanarito (GTO) (Weaver *et al.,* 2000), Pirital (PIR) (Fulhorst *et al.,* 1999), and Whitewater Arroyo (Fulhorst *et al.,* 2001), have demonstrated considerable genetic variation, in both nucleotide and amino acid sequences, among geographical and temporal isolates of the same virus species. At present, species designation for members of the genus *Arenavirus* is based on their antigenic differences; there are as yet no established genetic criteria for differentiating species. Recently, Bowen *et al.* (2000) proposed a cutoff value of 12% amino acid difference in the nucleocapsid (N) protein gene as a criterion for delineating arenavirus species. However, in phylogenetic studies of PIR virus strains isolated from rodents in central Venezuela, we have found an even greater degree of diversity (up to 16%) in the deduced amino acid sequence differences among viruses that are antigenically indistinguishable by complement fixation tests.

PIR virus, a member of clade A of the Tacaribe complex of New World arenaviruses, was originally isolated from a cotton rat, *Sigmodon alstoni,* collected in the community of Pirital, Portuguesa State, Venezuela in February 1994 during field studies on the epidemiology of Venezuelan hemorrhagic fever (Fulhorst *et al.,* 1997). During these studies, more than 4500 wild rodents were trapped from a variety of habitats within a six-state area covering approximately 75,000 km^2 in central Venezuela (Tesh *et al.,* 1999, 1993; Utrera *et al.,* 2000). More than 1000 arenavirus isolates were obtained from the rodents collected. A portion of the N protein gene from 30 selected PIR-like virus field isolates was subsequently am-

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plified, sequenced, and examined phylogenetically. The present paper identifies remarkable genetic diversity within a small region of Venezuela and reexamines the criteria for designating arenavirus species.

RESULTS

Pirital virus isolates

During our field studies on the epidemiology of Venezuelan hemorrhagic fever (VHF), more than 4500 animals, representing 10 different rodent species, were collected and tested for infectious arenaviruses. Because our objective was to sample animals that potentially might have contact with humans, the majority of these rodents were collected from agricultural and peridomestic habitats. In these habitats, *S. alstoni* was the most abundant species and constituted 48% of all rodents captured (Utrera *et al.,* 2000). PIR virus isolates were obtained from three different rodent species (*S. alstoni, Zygodontomys brevicauda,* and *Proechimys guairae*), but overall, the highest infection rates were found in *S. alstoni.* Virus isolation rates among field-collected *S. alstoni* were commonly in the range of 25–40% (Tesh *et al.,* 1999). Because of the large number of arenavirus isolates obtained, it was not feasible to characterize genetically each one; thus, the isolates were initially characterized as PIR-like or GTO-like, based on the results of immunofluorescence using specific mouse hyperimmune ascitic fluids prepared against the prototype PIR and GTO virus strains. After dividing the isolates into these two broad categories, selected representative of each group were sequenced. In all cases, the PCR results confirmed the IFAT results with regard to their genetic relationship to the prototype Pirital or Guanarito virus strains. Results of the genetic studies of the GTO-like isolates were published previously (Weaver *et al.,* 2000).

Sequence comparisons

Partial N protein gene sequences of the 28 rodent isolates of PIR virus, as well as those of strains VSV-488 and VAV-499 sequenced previously (Fulhorst *et al.,* 1997), were compared. All sequences were 616 nucleotides in length, excluding the PCR primers. Among PIR virus isolates, nucleotide sequences differed by up to 26%, with up to 16% deduced amino acid sequence differences. When compared to the published sequences of other New World arenaviruses, the PIR nucleotide sequences differed by 34–44%, and deduced amino acid sequences differed by 36–52%. By way of comparison, the other New World arenaviruses that have been sequenced (Table 1) differed by 24% (Machupo vs Junin) to 46% (Tacaribe vs Whitewater Arroyo) at the nucleotide level and 14% (Machupo vs Junin) to 54% (Tacaribe vs Whitewater Arroyo) in amino acid sequences.

TABLE 1

New World Arenaviruses Studied

Virus	Principal reservoir host	Known distribution	GenBank Acc. No.
Guanarito	*Zygodontomys brevicauda*	Venezuela	U43686
Pirital	*Sigmodon alstoni*	Venezuela	U62561
Tacaribe	*Artibeus sp.* (fruit bats)	Trinidad	M20304
Amapari	*Neocomys guianae*	Brazil	U43685
Flexal	*Oryzomys sp.*	Brazil	U43687
Sabia	unknown	Brazil	U41071
Junin	*Calomys laucha, C. masculinus*	Argentina	D10072
Oliveros	*Bolomys obscurus*	Argentina	U43248
Parana	*Oryzomys buccinatus*	Paraguay	U43689
Latino	*Calomys callosus*	Bolivia	U43688
Machupo	*Callomys callosus*	Bolivia	X62616
Allpahuayo	*Oecomys sp.*	Peru	AY012686
Pichinde	*Oryzomys albigularis*	Colombia	K02734
Tamiami	*Sigmodon hispidus*	Florida, USA	U43690
Whitewater Arroyo	*Neotoma albigula*	Southwestern USA	U52180

Phylogenetic studies

The aligned nucleotide sequences contained 650 characters, of which 432 were informative. Phylogenetic analyses, using maximum parsimony, neighbor joining, and maximum likelihood methods, yielded trees with very similar topology, differing primarily in the terminal relationships among a few PIR virus isolates. One notable discrepancy was the placement of Allpahuayo virus (Moncayo *et al.,* 2001), which appeared as a sister to Pichinde virus when deduced amino acid sequences were analyzed, or when nucleotides were analyzed using maximum likelihood. In contrast, Allpahuayo occupied a position basal to the Flexal–Parana clade in the trees generated with nucleotide sequences using neighbor joining and parsimony. Based on higher bootstrap values, amino acid trees of the 17 different New World arenaviruses were generally more robust than nucleotide trees; for example, the Allpahuayo–Pichinde amino acid sequence tree grouping had 64 and 84% bootstrap support with neighbor joining and parsimony, respectively, while the Allpahuayo–Flexal–Parana clade was supported by values of less than 50% in nucleotide analyses. These data suggested that the Allpahuayo–Pichinde grouping is more likely to be correct.

As in the previous analyses, PIR was positioned at the base of clade A (Bowen *et al.,* 1996), which included Tamiami, Whitewater Arroyo, Flexal, Parana, Pichinde, and Allpahuayo viruses. The PIR viruses comprised a monophyletic group in all trees, with bootstrap support of 100% using all methods (Fig. 2). Within the PIR group, the same 14 distinct lineages or genotypes (designated genotypes 1–14) were identified using all methods; these

TABLE 2

Pirital-like Virus Isolates Examined

Strain	Host	Location	Date	Genotype	GenBank Accession No.
1583	*S. alstoni*	Centro de Recria, Apure	MAR 1995	1	AF371441
1645	*S. alstoni*	Centro de Recria, Apure	MAR 1995	1	AF371442
1575	*S. alstoni*	Caño la Pica, Apure	MAR 1995	2	AF371440
1657	*S. alstoni*	Achaguas, Apure	MAR 1995	3	AF361443
1895	*Z. brevicauda*	El Baul, Cojedes	APR 1995	4	AF371446
2600	*Z. brevicauda*	Gato Negro, Portuguesa	OCT 1995	5	AF371450
2731	*S. alstoni*	Portachuelo, Portuguesa	NOV 1995	5	AF371451
3081	*S. alstoni*	La Espinalua, Portuguesa	MAR 1996	5	AF371453
3078	*S. alstoni*	La Espinalua, Portuguesa	MAR 1996	5	AF371452
2550	*Z. brevicauda*	Gato Negro, Portuguesa	AUG 1995	5	AF371449
488[a,b]	*S. alstoni*	Pirital, Portuguesa	FEB 1994	5	U62561
2508	*S. alstoni*	Los Cocos, Portuguesa	AUG 1995	5	AF371448
393	*S. alstoni*	Pirital, Portuguesa	JAN 1994	5	AF371454
4274	*S. alstoni*	Guanarito, Portuguesa	MAR 1997	5	AF371457
499[a]	*S. alstoni*	Pirital, Portuguesa	FEB 1995	5	U62562
4214	*S. alstoni*	Pirital, Portuguesa	FEB 1997	5	AF371455
4219	*S. alstoni*	Pirital, Portuguesa	FEB 1997	5	AF371456
1743	*S. alstoni*	Palo Seco, Guarico	MAR 1995	6	AF371444
452	*S. alstoni*	Ospino, Portuguesa	FEB 1994	7	AF371458
535	*S. alstoni*	Caño Hondo, Cojedes	MAR 1994	8	AF371460
4648	*S. alstoni*	Sarare, Lara	JUN 1997	9	AF371459
3945	*S. alstoni*	Caño Hondo, Cojedes	JAN 1997	10	AF371464
3876	*S. alstoni*	Caño Hondo, Cojedes	JAN 1997	10	AF371463
3367	*S. alstoni*	Gato Negro, Portuguesa	AUG 1996	11	AF371465
3673	*S. alstoni*	Gato Negro, Portuguesa	AUG 1996	12	AF371466
2148	*Z. brevicauda*	Papayito, Portuguesa	MAY 1995	12	AF371447
1881	*Proechimys* sp.	El Baul, Cojedes	APR 1995	13	AF371445
1895	*Z. brevicauda*	El Baul, Cojedes	APR 1995	13	AF371446
770	*S. alstoni*	Dolores, Barinas	APR 1994	14	AF371462
681	*S. alstoni*	Dolores, Barinas	APR 1994	14	AF371461

[a] Strains sequenced previously.
[b] Pirital virus prototype strain.

lineages differed by 6–26% in nucleotide and up to 16% in amino acid sequences, and each was supported by bootstrap values of 100%, with the exception of genotype 12 at a minimum of 74% support. When PIR virus sequences were examined using maximum likelihood methods, an estimate of 5:1 was obtained for the ratio of transitions to transversions, and a gamma value of 0.33 for substitution rate uniformity across nucleotide sites.

Of the 14 PIR virus genotypes identified, only three (5, 12, and 13) were sampled in more than one location; the remaining 11 genotypes were isolated in only one collecting location. When the PIR virus isolates were compared by locality, many of the different genotypes were sampled in only one region or locality (Table 2, Fig. 1). However, the sample size for most genotypes was quite small, with most represented by only one to two isolates. Genotype 5 was the most commonly sampled (12 isolates) and was sampled over the broadest geographic region in much of Portuguesa state. Several genotypes were isolated from only one rodent species, but sample sizes again were very small for most.

Genetic diversity of Pirital virus within infected hosts

To evaluate the genetic heterogeneity within PIR virus isolates, five individual clones were sequenced for five rodent isolates selected at random (strains 681, 1743, 2731, 4214, and 4219). Clones from these isolates had a mean of 0.23% nucleotide sequence variation vs each consensus sequence (range 0.05–0.32%). The expected variation, based on the maximum RT-PCR error rate estimated previously (Weaver *et al.,* 2000), was 0.06% for each clone. When this RT-PCR error rate estimate was subtracted from each mean, the PIR clones exhibited a mean of 0.17% sequence diversity vs the consensus sequences. In one extreme case, not included in the above calculations, an individual clone of strain 2731 (clone B) differed from the consensus sequence by 39 nucleotides. The phylogenetic placement of this clone sequence (Fig. 2) indicated that it represented a distinct lineage, suggesting a dual infection of this rodent. Because the strain 2731, clone B sequence differed by at least 10 nucleotides from that of all other PIR strains, including all of those made from samples processed on

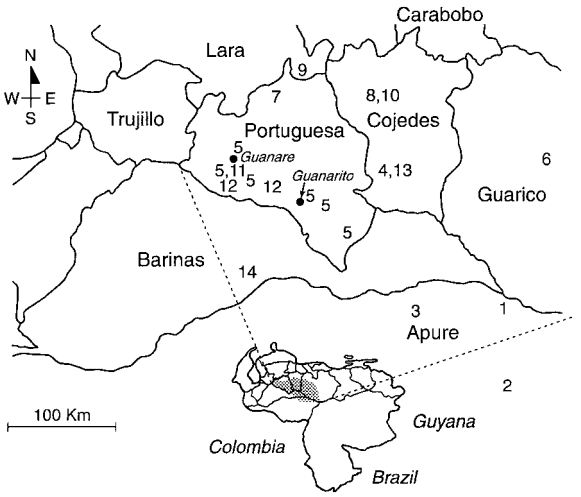

FIG. 1. Map of western Venezuela showing the locations where Pirital virus strains were isolated. The numbers refer to the Pirital virus genotype(s) found at each locality.

the same days as strain 2731, this isolate appears to represent a naturally mixed population containing two distinct genotypes representing distinct PIR virus lineages. For the remaining four strains examined, all clones grouped together when analyzed phylogenetically, suggesting quasispecies populations (Domingo, 1998; Holland and Domingo, 1998) generated during these or previous rodent infections. Another clone from strain 2731 included a single nucleotide deletion that resulted in a frameshift in the N ORF, resulting in a predicted truncation of the N protein.

Antigenic diversity

To assess whether the genetically diverse PIR viruses (Fig. 2) were also antigenically distinct, cross-CF tests were done using single-shot hamster immune sera prepared to six different PIR virus genotypes (PIR 488, genotype 5; PIR 535, genotype 8; PIR 1575, genotype 2; PIR 1743, genotype 6; PIR 1881, genotype 13; and PIR 2148, genotype 12). Results are given in Table 3. By CF tests, the six virus strains (genotypes) were antigenically indistinguishable.

For comparison, Table 4 shows the results of CF tests with antigen to the prototype strain of PIR virus (PIR 488) and HMAF prepared to PIR 488 and five other clade A, Tacaribe complex arenaviruses (Parana, Pichinde, Flexal, Whitewater Arroyo, and Allpahuayo). Using HMAF, which are more broadly reacting and less specific than the single-shot hamster sera, PIR virus still was easily distinguishable from the other five clade A viruses.

DISCUSSION

Sequence diversity and arenavirus systematics

A previous genetic study of GTO virus isolates from the same regions of western Venezuela demonstrated considerable diversity (Weaver *et al.,* 2000). These GTO strains differed by up to 17% in nucleotide sequences and up to 9% in deduced amino acid sequences. In the present study, we detected even more diversity among PIR virus strains: nucleotide sequences differed by up to 26%, with up to 16% deduced amino acid sequence differences. These levels of N protein gene sequence divergence are slightly greater than those exhibited by Lassa viruses in Africa, which differ by up to 24% at the nucleotide level and 12% at the amino acid level (Bowen *et al.,* 2000). However, the PIR viruses included in the present study were sampled from a smaller geographic region of Venezuela than the Lassa viruses, which were sampled from Nigeria, Sierra Leone, Liberia, and Guinea.

Somewhat surprisingly, CF tests with representatives of the most divergent PIR virus lineages provide no evidence for antigenic differences that would warrant dividing these viruses into separate species or even subtypes. By way of comparison, Machupo and Junin virus S gene nucleotide sequences differ by only 24%, and their N protein amino acid sequences differ by only 14%, yet these viruses are considered distinct arenavirus species. Machupo and Junin viruses are members of clade B of the Tacaribe complex (Bowen *et al.,* 1996). By CF tests, they also are indistinguishable (Casals *et al.,* 1975), although they can be differentiated by plaque reduction neutralization tests (Johnson *et al.,* 1967). This apparent discrepancy underscores a fundamental problem in arenavirus taxonomy: traditional taxonomic criteria emphasize antigenic comparisons using serological tests, yet some of the more recently described arenaviruses that have been sequenced have never been fully characterized antigenically. Biosafety and transport/import restrictions on work with many arenaviruses, as well as the lack of appropriate containment facilities to carry out traditional antigenic testing with many arenaviruses at most research institutions, will continue to limit antigenic analyses of these viruses. In contrast, genetic testing using inactivated RNA preparations from cell culture passages generated in high-security labs, or from tissues of field collected rodents, is highly feasible for most investigators. These circumstances will undoubtedly result in the continued preponderance of genetic data without complementary antigenic characterization of many arenaviruses. Unfortunately, the levels of sequence divergence consistent with species status, either nucleotide or amino acid, have not been well defined, so taxonomic difficulties will persist.

Based on sequences of different Lassa virus strains, Bowen *et al.* (2000) proposed a minimum cutoff value of 12% amino acid sequence divergence in the N protein for classifying distinct arenavirus species. However, the results of our genetic and antigenic studies indicate that amino acid sequence divergence within the N protein must exceed 16%, at least in some cases (e.g., PIR virus),

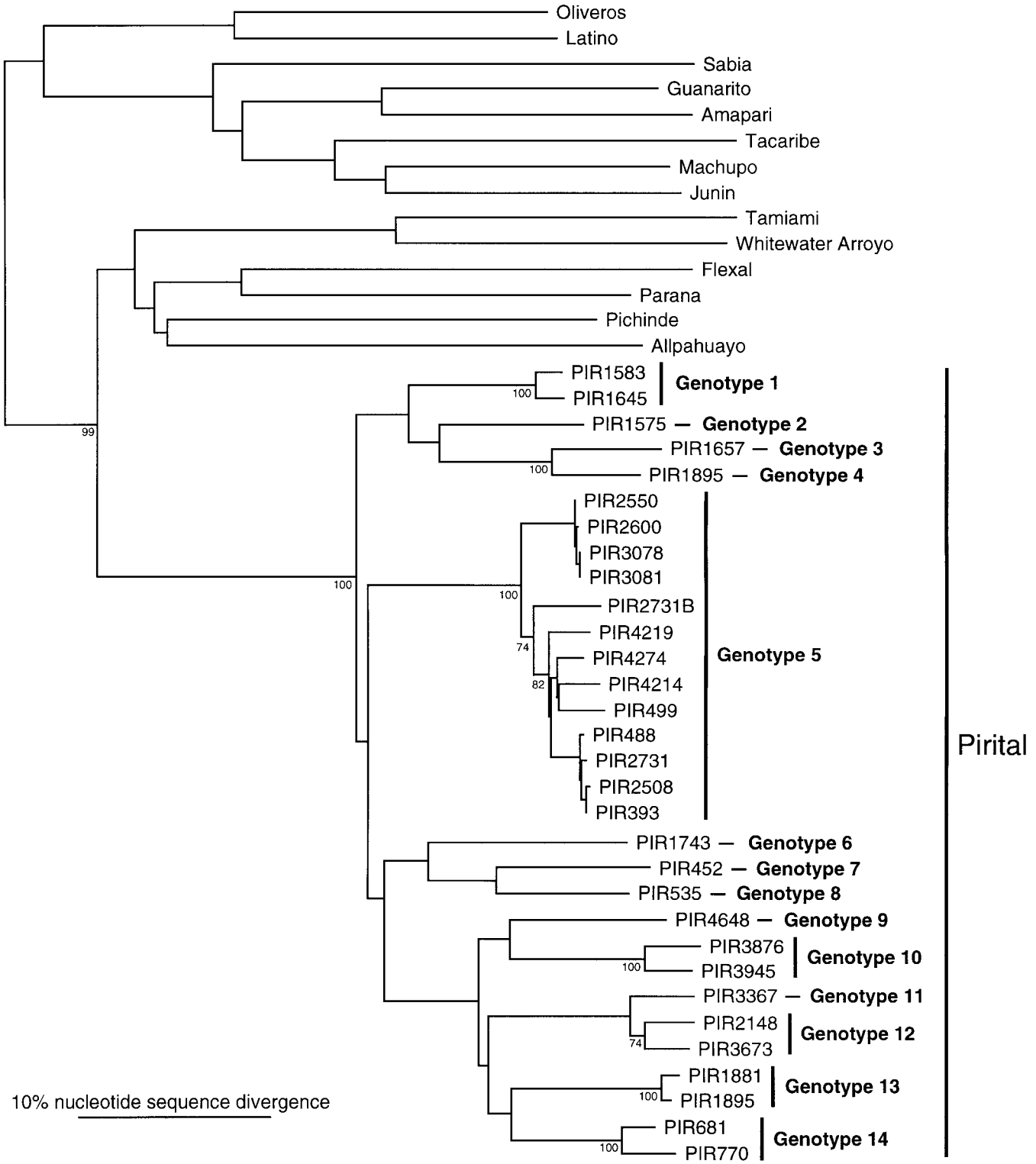

FIG. 2. Phylogenetic tree generated from nucleocapsid protein gene nucleotide sequences of 30 PIR virus isolates and homologous sequences of other New World Arenaviruses. This tree was generated using the neighbor joining program and was nearly identical in topology to the trees generated with maximum parsimony and maximum likelihood. The Allpahuayo–Pichinde grouping was constrained in the nucleotide analysis, based on more robust bootstrap support for this topology obtained using amino acid sequences. Numbers indicate bootstrap values for groups to the right of nodes. The scale shows a genetic distance of 0.1, generated using the Kimura two-parameter formula.

before antigenic differences consistent with species distinctions can be predicted. Additional analyses of sequence diversity within and sequence divergence among arenavirus species, combined with more thorough and comprehensive antigenic studies, are needed to arrive at sequence divergence values predictive of

TABLE 3

Results of Complement Fixation Tests with Six Different Genotypes of Pirital Virus

	Antibody						
Antigen	PIR488	PIR535	PIR1575	PIR1743	PIR1881	PIR2148	Control
PIR488	≥256/16[a]	48/16	96/16	96/16	96/4	96/16	0
PIR535	≥256/2	48/2	96/2	96/2	96/2	96/2	0
PIR1575	≥256/16	48/16	96/16	96/16	96/16	96/16	0
PIR1743	≥256/16	48/16	96/16	96/16	96/16	96/16	0
PIR1881	≥256/16	48/16	96/16	96/16	96/16	96/16	0
PIR2148	≥256/16	48/4	96/4	96/4	96/4	96/4	0
Control	0	0	0	0	0	0	0

Note. PIR488 (VAV-488) is the prototype strain of Pirital virus. Control antigen and antibody were normal mouse brain and mouse ascitic fluid, respectively.

[a] Reciprocal of highest antibody titer/reciprocal of highest antigen titer.

antigenic differences and useful for arenavirus systematics.

Pirital virus population partitioning

Remarkably, we detected 26% nucleotide sequence diversity among consensus nucleotide sequences in isolates spanning a range of less than 400 km. The factors that allow PIR viruses to be maintained in independent evolutionary lineages within such a small geographic range without competitive exclusion are not known. One possibility is that restricted mobility of the rodent hosts, *S. alstoni* and *Z. brevicauda,* allows independent virus lineages to circulate allopatrically without direct competition for hosts. Detailed studies to examine the gene flow of these rodent hosts are needed to test this hypothesis. Another possibility is that different PIR genotypes are partitioned by their rodent host species. Although our sample sizes were quite limited, the finding that genotype 5 was found only in *Z. brevicauda* at the Gato Negro site, while genotype 12 was found only in *S. alstoni* at the same location, supports this hypothesis. However, both genotypes 8 and 10 were isolated from *S. alstoni* at the Caño Hondo site, indicating that the niches of these two genotypes may not be distinct. Since the rodent species in which arenaviruses are isolated may not always be the true reservoir host in which long-term virus evolution occurs, arenavirus–host relationships need further study before these issues can be resolved.

Another factor that could influence arenavirus evolution and population partitioning is superinfection exclusion, either genetically or immunologically mediated. If only very closely related PIR virus genotypes are excluded from superinfection but not more distantly related genotypes, diversification could be favored evolutionarily. Our finding of a dual infection with strain 2731 and 2731B, both members of Genotype 5 (Fig. 2), could contradict this hypothesis. However, a dual infection could also have occurred simultaneously or in quick succession, before superinfection exclusion takes effect. Superinfection experiments with natural rodent hosts are needed to determine if exclusion occurs and to assess its possible effect on arenavirus evolution.

Genetic diversity within Pirital virus populations in individual hosts

The diverse quasispecies populations of PIR virus detected in rodents probably result from the persistent nature of infection in the natural rodent hosts (Childs and Peters, 1993; Fulhorst *et al.,* 1999), resulting in opportunities for extensive mutations and possibly selective (Darwinian) pressure for immune escape. For example, if immunity against the infecting antigenic variant is not effective against certain antigenic mutants (immune escape), those mutants will be favored evolutionarily during persistent infection. However, a more direct genetic analysis of viral RNA populations within individual rodents is needed to determine if the sequence diversity we detected reflects differences in the host compartment sampled, amounts of infectious virus amplified in cell culture, or possible selection for diversity within Vero cell cultures. Unfortunately, the original rodent samples from

TABLE 4

Results of Complement Fixation Tests with the Pirital Virus Prototype (PIR 488) and Five Other Clade A Viruses in the Tacaribe Complex

Antigen	Antibody pirital	Homologous
Pirital	64/16	
Parana	<8/8	≥64/16
Pichinde	<8/8	512/64
Flexal	<8/8	≥256/32
Whitewater Arroyo	<8/8	128/32
Allpahuayo	<8/8	512/64

[a] Reciprocal of highest antibody titer/reciprocal of highest antigen titer.

which our PIR viruses were isolated are not available for this kind of analysis. However, levels of genetic diversity similar to that which we detected in passaged rodent isolates have been reported in isolates of GTO virus (Weaver *et al.,* 2000) and several other RNA viruses that develop persistent infections (Cichutek *et al.,* 1992; Domingo *et al.,* 1998; Martell *et al.,* 1992). Genetic analyses of PIR virus populations generated during experimental rodent infections using defined or cloned virus populations are needed to evaluate the relationship between quasispecies diversity and persistent infection.

Of particular interest was the detection of phylogenetically distinct PIR sequences in one *S. alstoni* isolate. Although the original rodent tissue was not available for direct identification of the dual infection prior to passage, laboratory records and sequencing of all isolates made during the same day indicate that cross-contamination is an unlikely explanation. The simplest explanation for this result is a dual infection of this rodent with two PIR virus genotypes. Additional sequencing of other viral genes such as the GP would be useful in confirming this conclusion. In contrast, the monophyletic grouping of all clones from the other isolates indicates that the diverse rodent quasispecies populations that we detected were either generated from a common ancestor during the rodent infections or transmitted together in relatively large virus populations during initial rodent infection.

MATERIALS AND METHODS

Safety

Recommended biosafety methods were used in the field to minimize the risk of infection of workers with rodent-borne zoonoses (Mills *et al.,* 1995). All laboratory work with arenaviruses or potentially infected rodent tissues was carried out in biosafety level 3 or 4 laboratory facilities following standard procedures (U.S. Dept. of Health and Human Services, 1999).

Study area

Rodent collections were made in the western plains (llanos) of Venezuela at selected sites in the states of Portuguesa, Barinas, Apure, Lara, Cojedes, and Guarico (Fig. 1). The Venezuelan llanos are a vast inland plain covering about 140,000 km^2, bounded by the Andes mountains and the Venezuelan coastal range in the west and north, and by the Orinoco River in the south and east (Utrera *et al.,* 2000). Altitudes in the study area range from about 50 to 300 m. Approximately 80% of the surface area is covered by savanna; the remainder includes deciduous and semideciduous gallery forests. The climate of the region is tropical with mean annual temperatures ranging from 27 to 31°C, and annual rainfall varies from about 1000 to 2000 mm, with most of the precipitation occurring between May and November.

Rodent sampling

Rodent trapping was carried out between January 1994 and April 1998 at 35 different sites in the western llanos as part of epidemiological investigations of Venezuelan hemorrhagic fever (de Manzione *et al.,* 1998). A detailed description and analysis of the trapping program was published elsewhere (Utrera *et al.,* 2000). After collection, animals were euthanized with chloroform and, using clean forceps and scissors, a sample of spleen, lung, and heart blood was obtained immediately after death (Tesh *et al.,* 1993). Blood and tissue samples were placed in marked plastic cryovials on wet ice for transport to a field laboratory in Guanare, where they were transferred to liquid nitrogen freezers for storage. These samples were subsequently transported on dry ice to the Virology Unit of the National Institute of Hygiene in Caracas or to the Special Pathogens Branch of the Centers for Disease Control and Prevention, Atlanta for primary culture. Carcasses of animals were either preserved in 10% formalin and archived at the Museum of Natural Sciences, National Experimental University of the Llanos, Guanare, Venezuela or incinerated at the processing site.

Virus isolation and typing

Blood and/or spleen homogenates were inoculated individually into 25-cm^2 plastic flasks containing monolayer cultures of Vero E-6 cells (Fulhorst *et al.,* 1999; Tesh *et al.,* 1993). Cell cultures were maintained at 37°C and observed regularly for the presence of viral cytopathic effect (CPE). After 10 or 11 days of incubation (regardless of whether CPE was observed), cells were scraped from the flask and a drop of the suspension (ca. 20 μl) was placed on 12-well glass microscope slides (Cell-Line Associates, Inc., Newfield, NJ). After drying, the cells were fixed for 10 min in cold acetone and examined for the presence of arenavirus antigen by indirect fluorescent antibody technique (IFAT), using GTO and PIR hyperimmune mouse ascitic fluids (HMAF) in combination with a commercially prepared (Sigma, St. Louis, MO), fluorescein-conjugated, goat anti-mouse immunoglobin (Tesh *et al.,* 1994). Using the GTO and PIR HMAF at dilutions of 1:20, it was possible to separate the local arenavirus isolates included in this study into two broad groups (GTO-like or PIR-like) based on the intensity of fluorescence.

Immune reagents

The HMAF to GTO and PIR viruses were prepared in adult outbred Swiss mice, as described before (Fulhorst *et al.,* 1997; Tesh *et al.,* 1994). The immunogens used for preparation of the HMAF were crude brain homogenates (10% w/v in phosphate-buffered saline) of newborn mice inoculated intracerebrally with the prototype strains of

GTO (INH-95551) (Tesh *et al.,* 1994) and PIR (VAV-488) (Fulhorst *et al.,* 1997) viruses. The adult immunization schedule consisted of four intraperitoneal injections of the immunogen mixed with Freund's adjuvant, given at weekly intervals. Sarcoma 180 cells were given after the final injection to induce ascites formation (Fulhorst *et al.,* 1997). HMAFs to each of the following other arenaviruses were also prepared by the same method: Pichinde (strain CoAn 3739); Parana (strain 12056); Flexal (BeAn 293022); Whitewater Arroyo (strain AV9310135); and Allpahuayo (CLHP 2098).

Immune sera were also prepared in adult Syrian hamsters, *Mesocricetus auratus,* against six genotypically distinct PIR-like virus isolates (PIR-535, 1575, 1743, 1881, 2148, and the prototype 488). Each hamster was given a single, intraperitoneal or subcutaneous injection of 100 μl of an untitered stock of one of the six viruses, prepared from infected Vero cells. These "one-shot" immune sera were used in complement fixation (CF) tests.

Viral antigens

A single litter of newborn mice was inoculated intracerebrally with ca. 20 μl of each of the arenavirus stocks noted above to make antigens for use in CF tests. Infected brain material from sick or dead mice was treated by the sucrose–acetone extraction method (Beaty *et al.,* 1989) to prepare the antigens.

Complement fixation tests

CF tests were performed using a microtechnique (Beaty *et al.,* 1989), with two units of guinea pig complement. Titers were recorded as the highest dilution giving 3+ or 4+ fixation of complement.

Genetic characterization of selected Pirital virus isolates

Twenty-eight PIR-like virus isolates, representing a variety of rodent hosts and geographic localities, were selected from more than 1000 arenavirus isolates recovered. For comparison, we also included two isolates previously sequenced, VAV-488 and VAV-499 (Fulhorst *et al.,* 1997). Identification of the 30 virus isolates by strain number, source, geographic origin, and collection date is given in Table 2.

A phylogenetically informative portion of the nucleocapsid (N) protein gene described previously (Bowen *et al.,* 1996) was amplified and sequenced as reported earlier (Weaver *et al.,* 2000). RNA was extracted from first-passage Vero cell culture supernatants or cells 7–10 days after infection with rodent tissue homogenates. RNA was extracted from 0.25 ml of the supernatant using Trizol LS (BRL Laboratories, Bethesda, MD), or Trizol was added directly to infected cell monolayers, and RNA was extracted and purified according to the manufacturer's protocol. The cDNA was synthesized using primer 19C (Bowen *et al.,* 1996) and Superscript reverse transcriptase (BRL Laboratories) according to the manufacturer's protocol, and a PCR was carried out as described previously using primers 1696R or NW1696R and 1010C (Bowen *et al.,* 1996, 1997). Amplicons were visualized on 1.0% agarose gels stained with ethidium bromide, and bands corresponding to the predicted 664-bp products (including primers) were excised and purified using the QIAquick gel extraction kit (Qiagen, Santa Clarita, CA) according to the manufacturer's protocol. Purified amplicon DNA for each strain was sequenced directly with primers 1010C and 1696R, and amplicons from some strains were also cloned into the PCRII plasmid vector (Invitrogen, Carlsbad, CA) for sequencing with vector-specific primers. Sequencing was performed using the Applied Biosystems (Foster City, CA) Prism sequencing kits and a Model 377 automated sequencer. When sequences of individual clones differed, consensus sequences were determined by comparison with direct amplicon sequences.

Phylogenetic analyses

Sequences were aligned using the PILEUP (Devereux *et al.,* 1984) software implemented in the Wisconsin Package version 8.0. For alignment of different arenavirus sequences, deduced amino acid sequences were first aligned using the default parameters, and nucleotide sequences were aligned manually to preserve proper codon homology. Phylogenetic analyses were conducted using maximum parsimony, neighbor joining, and maximum likelihood programs implemented in the PAUP 4.0 software (Swofford, 1998), as well as the fastDNAml maximum likelihood program (Olsen *et al.,* 1994). For some parsimony analyses, transversion:transition weighting of 5:1 was used to increase the accuracy of internal groupings (Hillis *et al.,* 1994), based on maximum likelihood estimates; for distance analyses, the Kimura two-parameter and F84 formulas were used (Swofford, 1998). Maximum likelihood analysis included empirically determined nucleotide frequencies and a proportion of invariant nucleotide sites estimated at 0.16 based on the assumption that amino acids conserved across the partial N sequences of all arenaviruses are not free to vary and based on the number of nondegenerate sites within these codons. Bootstrap analyses were conducted with 1000 resamplings of the original data set (Felsenstein, 1985).

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